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Use of the TRPV1 Agonist Capsaicin to Provide Long-Term Analgesia in a Rat Limb Fracture/Open Repair, Internal Fixation Model

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14. ABSTRACT
Traumatic orthopedic injuries comprise a large portion of the injuries that are seen in our military servicemen in Operation Iraqi Freedom and Operation Enduring Freedom. Previous to this study there had been only one animal model for acute long bone fracture pain described. This model was useful in studying fracture pain by itself, but due to the method of fracture stabilization, the model did not follow real-world circumstance for injury followed by repair. During this period of research we have successfully developed and tested a novel rat pain model for acute traumatic femoral fracture followed by repair via intramedullary nail fixation that closely mimics what happens in real-world situations. Our model is consistent and reproducible and will help facilitate the discovery and evaluation of novel pain relief techniques that could benefit care of our wounded warriors as well as civilian traumatic injuries. Using our model, we could not show an pain relief benefit of injecting capsaicin around the fracture site.

15. SUBJECT TERMS
Femur fracture, Rat Model, Pain, Capsaicin, Trauma, TRPV1

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Introduction

The acute management of pain in the setting of traumatic extremity injury with bone fractures is an integral part in the care of many of the casualties in the current Areas of Responsibility, specifically from Operation Iraqi Freedom (OIF) and Operation Enduring Freedom (OEF). These injuries are associated with substantial acute pain that is often difficult to treat and may lead to the development of chronic pain syndromes that hamper the wounded soldier's ability to rehabilitate, return to duty, or even to reintegrate into society. The current methods for treating acute pain associated with extremity injuries rely most heavily on opiates given either intravenously or orally. While these agents are useful in the treatment of acute pain, they have serious side effects like respiratory depression and sedation that limit their use especially in forward military locations. In addition, opiate use for pain control can lead to long term opioid tolerance, dependence, and addiction. Most alarmingly, opiate use has been associated with the development of hyperalgesia, leading to a state where the patient may experience pain even at sites distant from the initial injury.[10,15,23,30]

As our ability to adequately treat acute pain is not yet sufficient, even in our modern facilities,[3] much focus has been given to find alternate modalities for acute pain control. Recently, a novel pain receptor site called the transient receptor potential vanilloid type I (TRPV1) has been targeted as a potential site of analgesic therapy. The TRPV1 receptor is a non-selective cation channel that is widely expressed in neuronal tissue and is present on the nociceptive Aδ and C nerve fibers.[34] The TRPV1 channel is important in the development and mediation of neurogenic pain and inflammation, chronic pain, and even may play a role in opioid induced hypersensitivity.[22,33] TRPV1 expression and sensitivity on the cell membrane is modulated by proinflammatory cytokines such as prostaglandin E2 (PGE2), prostacyclin (PGI2), protein kinase C (PKC) and protein kinase A (PKA), as well as by nerve growth factor (NGF) and neurotrophic factor (NTF), among others.[25,28,31]

TRPV1 is activated by the naturally occurring substance, capsaicin, as well as by its analog resiniferatoxin (RTX). Activation of TRPV1 by capsaicin and RTX leads to brief depolarization of the nerve followed by a prolonged inhibition of the receptor leading to blockage of noxious stimulus propagation along sensory nerves and analgesia that may last many weeks[13] while sparing motor and tactile sensory function.[5,20,21,26,27] TRPV1 inactivation has been shown to prevent neurogenic inflammation,[14] and development of distant hyperalgesia induced by inflammation[19] and by nerve injury.[20] Numerous studies have shown the ability of the TRPV1 agonists capcaicin and resiniferatoxin to reduce or eliminate neuropathic pain, hyperalgesia, and pain related to soft tissue injury by either local or perineural infiltration.[17,18,20,21] Additional studies have shown an analgesic benefit in humans using purified capsaicin injection.[7,8,9,29] Moreover, unlike traditional local anesthetics, it seems that both capsaicin and resiniferatoxin have no apparent deleterious effects on nerve tissue[21] or bone healing at low dose.[22]

Recently, lower extremity fracture pain models have been described in two rodent models.[11,24] Both of these models are limited for their use studying the types of injuries incurred in combat as the fracture and surrounding soft tissue injuries are highly modified by having an intramedullary rod in place prior to the fracture. In real world combat injuries, much of the damage produced during the injury is due to deformation and displacement of the bone and subsequent injury to surrounding nerves, vessels, and soft tissues. To our knowledge, there are no published pain models for lower extremity fracture followed by fixation. In addition, there have been no published works evaluating the efficacy of locally applied capsaicin for analgesia in fracture pain or its effects on bone healing and local inflammation.

Body

Approved SOW

	Q1	Q2	Q3	Q4	Q5	Q6	Q7	Q8
Specific Aim #1 -- Create a rat pain model for lower extremity fracture with open reduction and internal fixation (ORIF).								
Task #1 -- Development of rat lower extremity fracture ORIF model								
1.a -- IACUC approval of protocol	UTHSCSA							
Milestone #1 -- Animal use protocol approval.								
1.b -- Test fracture model and open surgical repair: 10 rats	UTHSCSA	UTHSCSA						
Milestone #2 -- Open fracture repair model achieved								
Task #2 -- Test pain related behaviors in rat fracture model								
2.a -- Fracture and surgical open repair/internal fixation and behavioral testing on 40 Rats		UTHSCSA	UTHSCSA					
2.b -- Sacrifice and Histopathologic/Radiologic study of repaired fracture site		UTSHCA	UTHSCSA	UTHSCSA				
Milestone #3 -- Pain-related behaviors characterized in rat lower extremity ORIF model								
2.c-- Data analysis and manuscript preparation.			All Institutions					
Milestone #4 -- Publication of manuscript on rat pain model for lower extremity fracture with ORIF.								
	Q1	**Q2**	**Q3**	**Q4**	**Q5**	**Q6**	**Q7**	**Q8**
Specific Aim #2 -- Examine the dose response relationship of capsaicin infiltration to the soft tissue and fracture site to pain, inflammation, and fracture heali								
Task #1 -- Testing of rats by inducing fracture model and applying different concentrations of capsaicin								
1.a --IACUC protocol approval				UTHSCSA				
1.b --Injure and surgically repair and treat rats followed by behavioral testing.				UTHSCSA	UTHSCSA	UTHSCSA		
1.c --Sacrifice and perform histopathologic and radiologic study of treated rats				UTSHCA	UTSHCA	UTHSCSA	UTHSCSA	
1.d -- Data analysis and interpretation. Evaluate need for additional capsaicin dosing					UTSHCA	UTHSCSA	UTHSCSA	
1.e -- Data analysis and manuscript preparation						All Institutions		
Milestone #5 -- Publication of manuscript on the affect of capsaicin treatment on acute pain, inflammation, and bone healing in our rat model.								

Creation of a Rat Femur Fracture/Repair Pain Model:

All Procedures were approved by the Institutional Animal Care and Use Committee at the University of Texas Health Science Center San Antonio and the Animal Care and Use Review Office of the US Army Medical Research and Material Command and were in accordance with the guidelines of the National Institutes of Health for care and use of laboratory animals. Animals were housed in individual cages in conventional facilities with a 12 hour light/dark cycle and food available ad libitum. Animals were excluded from the study if the fracture produced was an oblique, comminuted, or too distal of a fracture, or if the fracture was not able to be reduced and fixed via an intramedullary nail.

Methods: 30 male Sprague-Dawley rats were divided into five groups; Fracture/Repair (FR), Pin-Only (PO), and Control (C). All animals received an intraperitoneal injection of 100mg/kg ketamine and 10 mg/kg xyalazine for the induction of anesthesia prior to any procedure being performed. For animals in Group FR, a traumatic midshaft femoral fracture was produced in the left leg of the animals using a three-point impact device (BBC Specialty Automotive Center, Lindon, NJ) as described by Bonnarens and Einhorn and subsequently adapted by Simon *et al*. The fracture was confirmed by radiography. Immediately following fracture, a 1mm incision was made over the greater trochanter and a 1.2mm kirschner wire of 32mm length was percutaneously placed through the intramedullary space in an anterograde fashion from the intertrochanteric notch to the distal femur. Confirmation of correct pin placement and reduction of the femur was assessed by radiography. (Figure 1) The incision was closed using 3.0 nylon suture and liquid adhesive. In Group PO, an anterograde intramedullary nail was placed as described above without creating a femoral fracture. Correct intramedullary nail placement was confirmed by radiography. Group C received only a sham incision over the greater trochanter. All animals were survived after surgery and tested for pain behavior using an incapacitance meter and guarding scores for 28 days. After testing on day 28, the animals were anesthetized with 3% Isoflurane and euthanized and both femurs harvested for micro CT and histologic analysis.

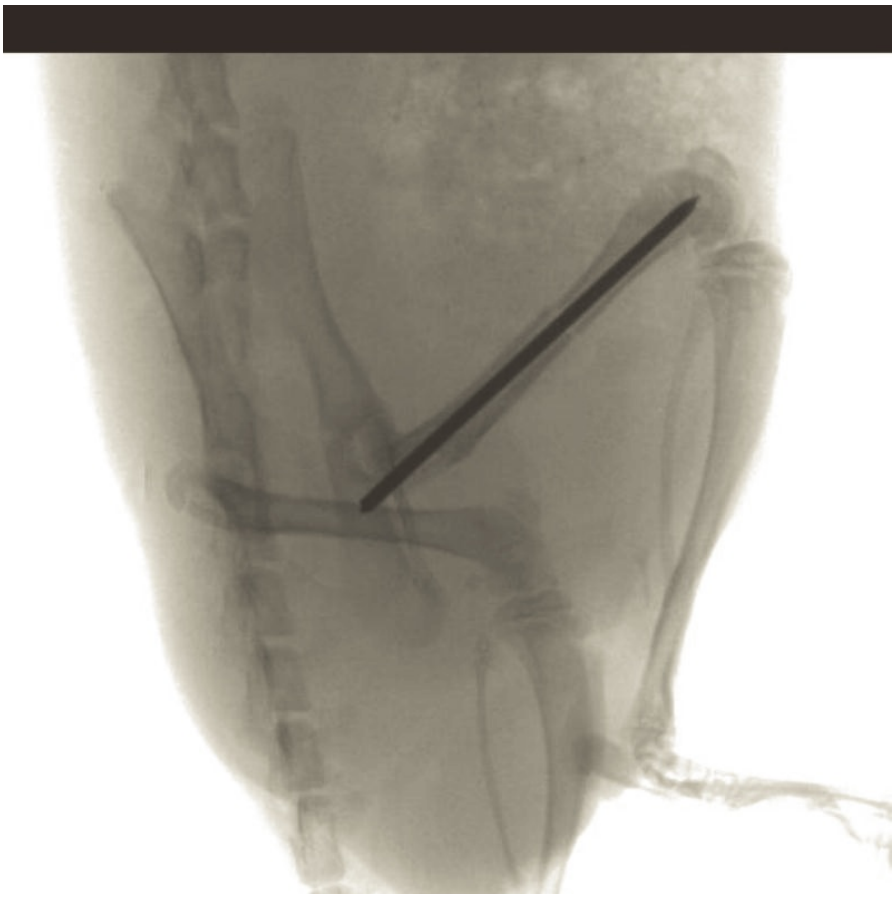

Figure 1: Lateral x-ray of fractured left femur with intramedullary nail.

Behavioral Testing: Animals were tested using an incapacitance meter (IITC Life Sciences, Woodland Hills, CA) and guarding scores before surgery (Day 0) and on post-operative days 1,2,4,7,10,14,21,and 28. Incapacitance scores were obtained by measuring the weight bearing on each hind limb averaged over 3 seconds for five consecutive trials. Incapacitance scores are reported as a percentage of weight bearing of the injured left leg of total weight bearing ([left/(left-right)] * 100). Guarding scores were assessed by observing the animal on a mesh stand for a period of 1 hour and recording the level of guarding every five minutes. Guarding scores were defined as 0= no guarding as evidence of blanching of the hind paw on the mesh, 1= hind paw lightly touching the mesh, but no blanching, and 2= hind paw not touching the mesh. The score was reported as a sum of all the scores over 60 minutes.

20 additional animals were randomized into two groups of 10 animals each to test for the effect of systemic analgesics on behavioral testing in our model. All animals had a femur fracture/repair as described above with behavioral testing on POD # 0,1,2,4,5, 6, and 7. On POD # 4,5, and 6 animals in Group Morphine (Group M) were tested using behavioral tests described above and then were given incremental doses of subcutaneous morphine (1mg/kg on day #4, 5mg/kg on day #5, and 10mg/kg on day #6). 30 minutes after giving the morphine, the animals were behavioral tested again. Animals in the control group received subcutaneous injections of saline and were tested in the same way as Group M. The person performing the behavioral testing and administering the subcutaneous injections was blinded to the animal groups.

Imaging: Femora were scanned with a desktop micro-computed tomography system (1172, SkyScan, Kontich, Belgium). Scans were collected with the specimens immersed in ethanol and using the following parameters: 60 kV tube voltage, 167 μA current intensity, 1336 x 2000 pixel matrix and a 20 μm^3 nominal isotropic resolution. Volumes of interest for the fractured femurs were centered on the fracture line and extended 3 mm above and below this image slice (Figure 2). Volumes of interest boundaries or contours were manually defined and included only the native cortical bone. Total bone volume (mm^3) was assessed within this volume. Next, this native cortical bone VOI was used to create an image mask that separated the native and

callus bone tissue. The image mask was created by subtracting the region encompassing the native cortical bone from the total region. Total bone volume and callus bone volume fraction (%) were assessed within the callus volume of interest (Figure 3). Contralateral femurs (right side) were scanned with the same parameters and analyzed to provide an internal contralateral control. Control limb volumes of interest were centered on the 55% of the length slice, as defined by the distance between the lesser trochanter and the distal growth plate. The length of this VOI was 6mm.

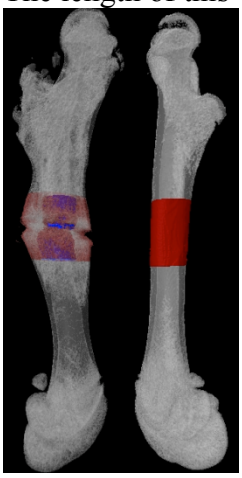

Figure 2: MicroCT imaging of normal (right) and fractured (left) femurs. The red shows the volume of interest used in data gathering.

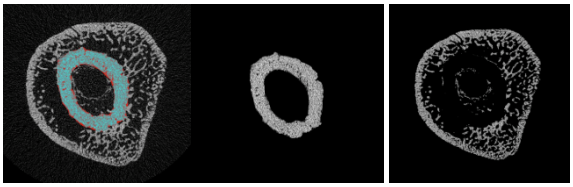

Figure 3: MicroCT imagine of fractured femur. Far left picture shows a cross section of the entire femur 28 days after fracture/repair. The middle image shows the native cortical bone inside of the fracture callus. The far right image shows the fracture callus with the cortical bone subtracted. The images were used to calculate the total bone volume and callus bone volume fractions.

Pathology: The left femurs of 6 rats were fixed in ten percent neutral buffered formalin, processed by conventional methods, embedded in paraffin, sectioned to 5 micrometers, and stained with hematoxylin and eosin. Sections were analyzed for the composition of the callus. Because it is possible for the position of the bones with respect to each other, to affect the healing process, whether or not the bones were aligned was also recorded. Bones were considered to be aligned if the lateral displacement was less than the width of one cortex.

Results: One animal was excluded due to a comminuted/oblique fracture. Mean scores for incapacitance meter across all testing days were 49.8+/- 2.5% for Group C, 34.7 +/- 2.3% for Group FR, and 36.2+/-2.2% for Group PO. Groups FR and PO had significantly worse mean incapacitance scores than group C ($p=0.001$). Groups FR and PO had no difference in mean incapacitance scores ($p=1$). Guarding score means were 1.1+/-0.9 for Group C, 9.7+/-0.8 for Group FR, and 8.8+/-0.8 for Group PO. Groups FR and PO had significantly worse mean guarding scores than group C ($p<0.001$). Groups FR and PO had no difference in mean guarding scores ($p=1$). When comparing individual test days, Group FR had significantly worse incapacitance meter scores than Group C on days 1,2,4,7, and 10 ($p<0.003$) and worse guarding scores on days 1,2,4,7, and 10 ($p<0.03$). Group PO had worse incapacitance scores than Group C on days 1,2,4 and 7 ($p<0.02$) and worse guarding scores on days1,2,4,7,10,14, and 21 ($p<0.03$). Group PO had worse guarding scores than Group FR on day 2 only ($p=0.003$). Incapacitance scores had no significant difference on any day between groups PO and FR. (Figures 4,5)

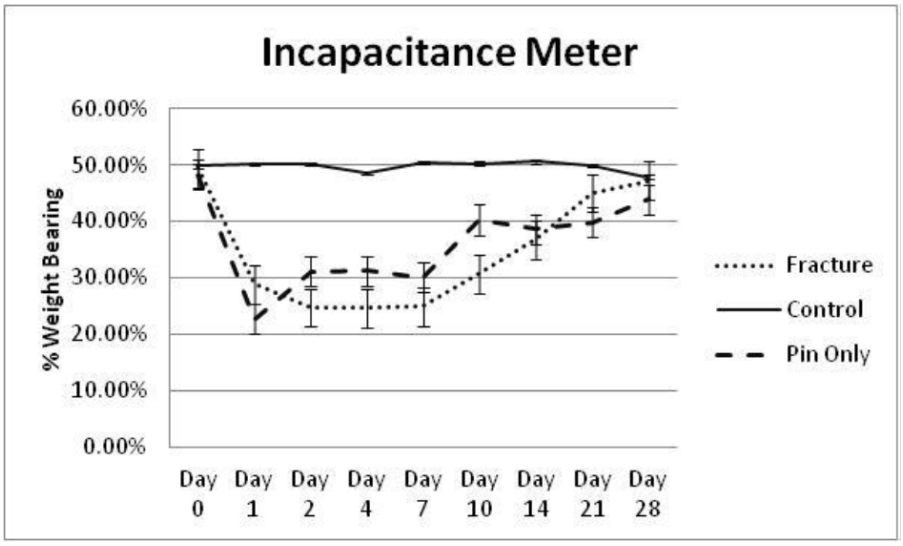

Figure 4: Incapacitance meter scores represented as percent weight bearing of left (injured) leg calculated by [left/(left+right)]*100.

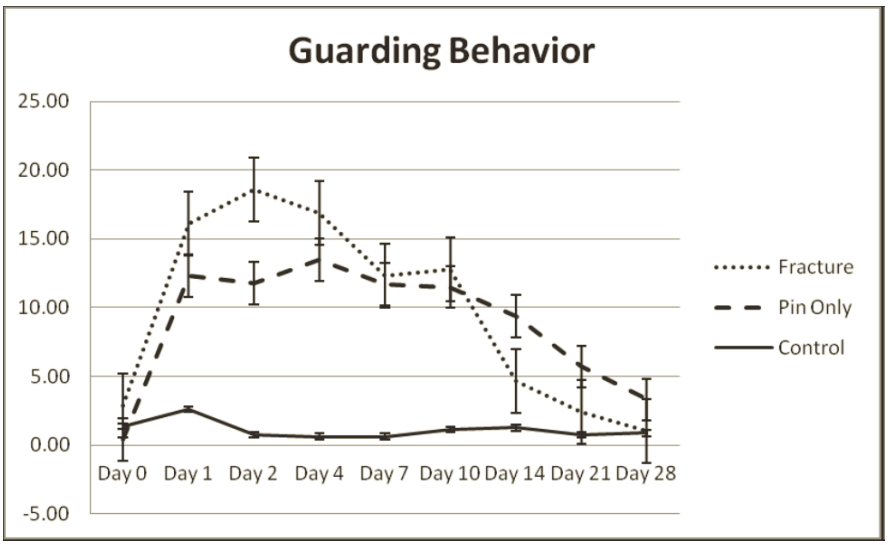

Figure 5: Guarding behavior of left (injured) leg.

In the morphine comparison, Guarding scores were significantly better after receiving morphine (Group PM mean 1.9 +/- 1.4) compared to the pre-morphine test (Group AM, mean 8.9 +/- 4.5) and the saline group (Group C, mean 7.7 +/- 2) on days 4,5, and 6 ($p<0.001$). (Figure 6) Incapacitance scores trended towards improvement in Group PM on days 4,5, and 6, but did not reach significance ($p=0.107$). (Figure 7)

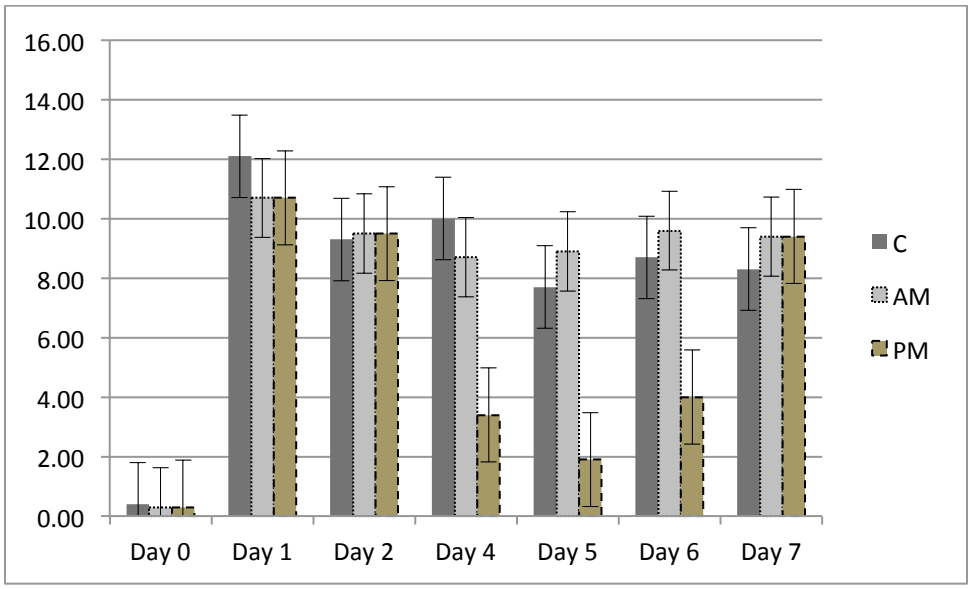

Figure 6: Guarding scores for morphine treated animals. C=control group. AM=testing before the animals received morphine. PM=testing after animals received morphine. Morphine was given on day #4 (1mg/kg), #5 (5mg/kg), and #6 (10mg/kg).

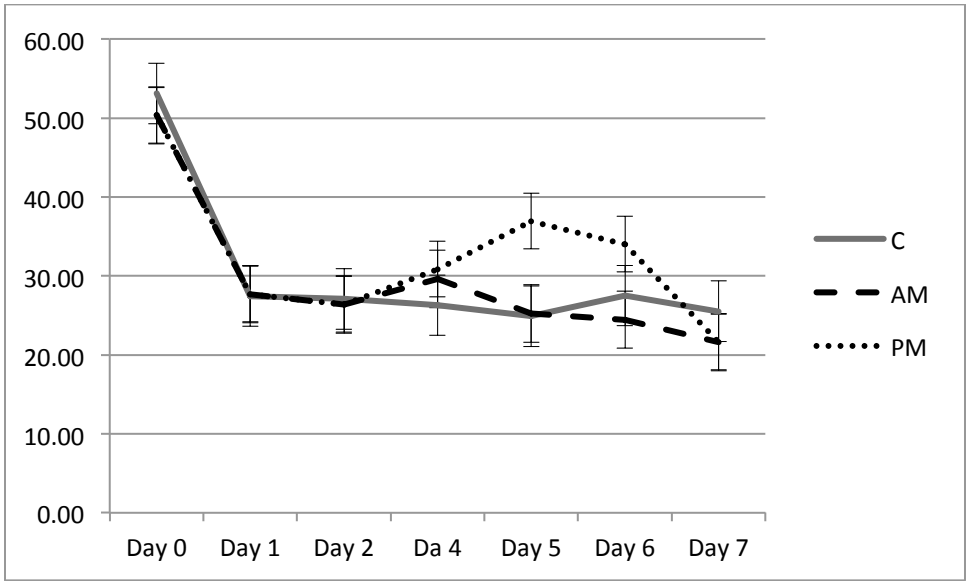

Figure 7: Incapacitance Meter for morphine treated animals. C=control group. AM=testing before the animals received morphine. PM=testing after animals received morphine. Morphine was given on day #4 (1mg/kg), #5 (5mg/kg), and #6 (10mg/kg).

Conclusion: We were able to successfully develop a traumatic femur fracture/repair pain model in the Sprague-Dawley rat. The model shows significant pain behavior that is consistent, reproducible, and predictable. Pain behavior testing showed a peak in pain scores between POD 2-4 with improvement towards baseline by POD 28. Interestingly, pain behavior in animals that received an intramedullary pin without fracture had similar pain behavior as the fracture/repair group in both severity and duration. Guarding behavior demonstrated a more robust improvement to systemic analgesics than did Incapacitance Meter behavior. Bone healing in our model results in a stable femur by 28 days post-fracture with virtually identical strength as non-injured femurs as measured by Mean Polar Moment of Inertia using micro-CT data. This model will be useful to test novel analgesic drugs and modalities that may prove valuable in treating human traumatic fracture pain like that seen in many of the injuries incurred by our deployed soldiers.

Capsaicin as an Analgesic for Traumatic Fracture Pain:

Methods: After creating and validating the rat femur/fracture repair pain model, we used the model to test the analgesic effects of various concentrations of capsaicin injected around the fracture site immediately after fixation. After IACUC approval as described above, 30 rats were randomized into three groups of 10 animals; Group 0.08% (200 mcg capsaicin), Group 0.04% (100 mcg capsaicin), and Group Placebo (Group P). All animals received a femur fracture and repair as described above. Immediately after verification of adequate reduction and fixation, 0.25ml of either 0.08% capsaicin solution (Group 0.08%), 0.04% capsaicin solution (Group 0.04%) or saline (Group Sa) was injected percutaneously into the site of the fracture. Animals were then recovered and tested for pain behaviors as described above. At the end of 28 days after the procedure, animals were euthanized and femurs harvested as described above for micro CT analysis.

Results: There was no difference in incapacitance test scores between any group at any time point ($p>0.05$) (Figure 8). Guarding scores were significantly different between Group 0.08% and Group Sa on day 10 only ($p=0.03$) and between Group 0.04% and Group Sa on day 10 ($p=0.026$). On all other days, there was no significant difference between any groups (Figure 9).

Histologic analysis showed no difference in callus formation or bone healing between any groups. MicroCT scan showed no difference in Mean Polar Moment of Inertia (measure that correlates to fracture strength) between groups.

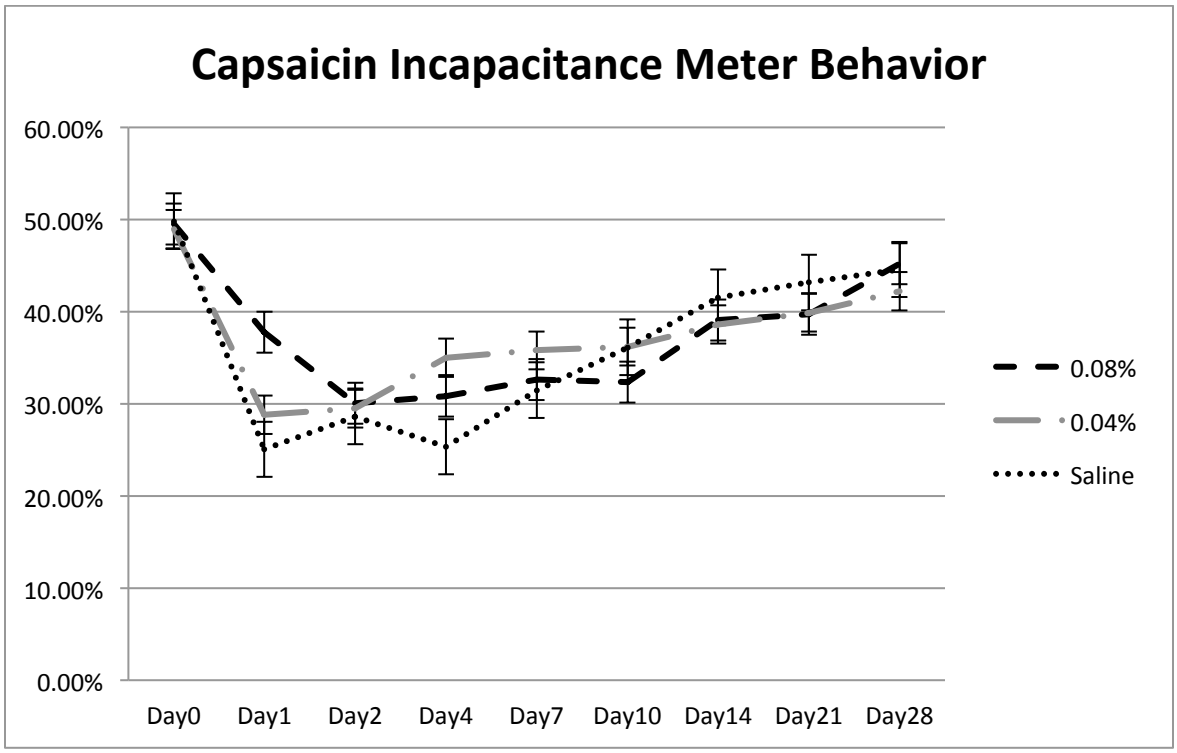

Figure 8: Incapacitance meter scores for Group 0.08% capsaicin, Group 0.04% capsaicin, and Group Saline. Standard error bars shown.

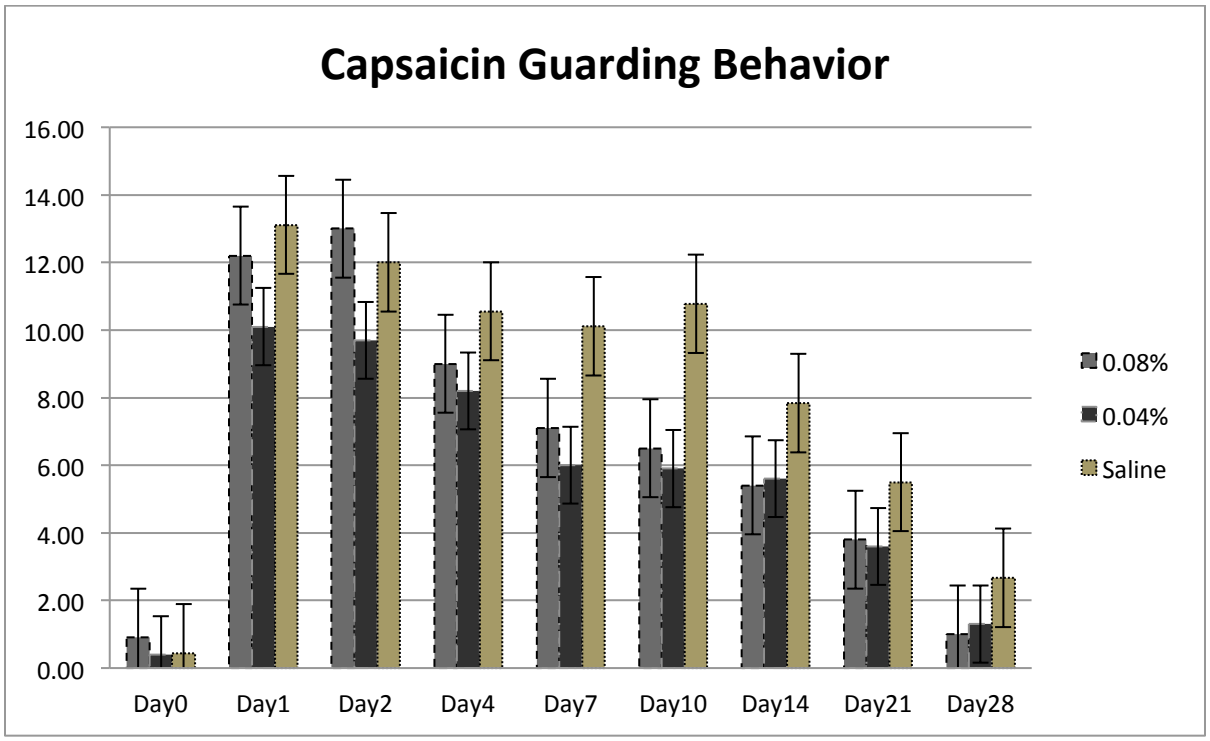

Figure 9: Guarding behavior for Group 0.08% capsaicin, Group 0.04% capsaicin, and Group Saline. Standard error bars shown.

Conclusion: Using our femur fracture/repair model, there does not appear to be a significant analgesic effect of injecting capsaicin around the fracture site after an acute femur fracture and repair by intramedullary nailing. These findings may be confounded by the amount of pain behavior seen in the pin only group as the capsaicin wouldn't be expected to have relieved any of the pain from the placement of the pin. A model that does not exhibit pain from the placement of an intramedullary nail might be helpful in determining whether or not local capsaicin injection around the fracture site can reduce fracture pain. However, in real world situations, femur fractures are most often repaired by placement of an intramedullary nail similar to our pain model.

Capsaicin in the concentration and dose tested by us does not seem to interfere with bone healing.

Key Research Accomplishments

- A novel rat fracture model has been developed that more closely resembles the timeline and methods of the injury/repair process of a femur fracture in humans. Unlike other small animal fracture/repair models, this model can produces and repairs the fracture at the same time whereas previously described models take up to 30 days to place the pin and then produce the fracture.
- We have validated our fracture/repair model as a pain model useful for studying fracture pain.

Reportable Outcomes:

- "A Novel Fracture Pain Model in the Rat" poster presented at the Spring 2012 American Society of Regional Anesthesia meeting and abstract published in *Reg Anesth Pain Med* Spring 2012 supplement.
- PI received an appointment as an assistant professor at the University Of Utah College Of Medicine after separating from Active Duty due largely to the work done as part of this grant.
- PI received an additional grant to study neuropathic pain that was received because of skills and knowledge obtained through working with the PI's mentors as part of the Career Development award from this Grant

Personnel Receiving Pay from Research Effort:

1. Krysten Chapa
2. Ajit Naik
3. Kun Zhang

Conclusion

We have successfully developed a novel traumatic femur fracture/repair pain model in the rat. Pain behaviors were robust with this fracture model and should prove useful in the testing of diverse analgesic modalities for acute fracture pain. Due to the pain behavior changes caused by intramedullary nail placement, this model would be best suited for systemic analgesics or regional analgesic techniques that would cover both the fracture area and the pin placement site. It is our belief that this model will help facilitate the development of novel analgesic modalities that will result in better pain relief for traumatic injuries in both our military and civilian populations.

Injection of capsaicin around the site of fracture did not appear to offer significant pain relief in our fracture model.

Career Development

The PI maintained a schedule allowing him at least 2 days/week dedicated to research during the course of the award with the exception of a 6 month deployment to Afghanistan. During that time, the PI has worked with his mentor, Dr. Brennan, at the University of Iowa to learn methods for animal pain behavior testing, small animal surgical procedures, as well as general study design and execution. The PI has been directly involved in the ongoing research performing all of the surgical procedures and directly overseeing the behavioral testing and care of the animals. In addition, the PI has been able to use his dedicated research time to acquire additional grant funding from the Air Force Surgeon General that funds a study of a novel use of a neuropathic analgesic drug. Because of his research efforts, the PI was named as the Assistant Program Director over Research for the combined Air Force/Army anesthesiology residency program in San Antonio where he mentored residents in training on their research projects and increased resident research output by 100%. As a direct result of the work and career development supported by this grant the PI was recruited by many academic anesthesia departments and joined the University of Utah department of anesthesia as an assistant professor where he continues his research to find novel methods of relieving pain for our wounded warriors.

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